

NUCLEIC ACID SEQUENCES THAT CAN BE USED AS PRIMERS AND PROBES
IN THE AMPLIFICATION AND DETECTION OF SARS CORONAVIRUS

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The present invention is related to nucleic acid sequences that can be used in the field of virus diagnostics, more specifically the diagnosis of infections with a novel human coronavirus causing Severe Acute Respiratory Syndrome (SARS).

10 An outbreak of atypical pneumonia, referred to as SARS, was first identified in late 2002 in Guangdong Province, China (Marra et al. (1) or Rota et al. (2)). After similar cases were detected in patients in Hong Kong, Vietnam, and Canada during February and March 2003, the World Health Organization (WHO)
15 issued a global alert for the illness. By May 22nd, 2003, over 8000 SARS cases and 682 SARS-related deaths were reported to WHO from over 30 countries around the world.

Most of these cases were related to exposure to SARS patients in residential or healthcare settings. The incubation
20 period for the disease usually ranges from 2 to 7 days. Infection is usually characterized by fever and flu-like symptoms, which are followed a few days later by a dry, non-productive cough, and shortness of breath. Death from progressive respiratory failure occurs in about 3% to nearly
25 10% of cases (3-7).

Conventional diagnostic kits could authorize the detection of antibodies produced in response to the SARS coronavirus infection. Different types of antibodies (IgM and IgG) appear and change in level during the course of infection. They can be
30 undetectable at the early stage of infection. Immunoglobulin G usually remains detectable after resolution of the illness. The following test formats are being developed, but are not commercially available yet:

- Enzyme Linked ImmunoSorbant Assay (ELISA): a test detecting a
35 mixture of IgM and IgG antibodies in the serum of SARS patients yields positive results reliably at around day 21 after the

onset of illness, and

- Immunofluorescence Assay (IFA): a test detecting IgM antibodies in serum of SARS patients yields positive results

5 after about day 10 of illness; this test format is also used to test for IgG; this is a reliable test requiring the use of fixed SARS virus infected cell cultures, studied under an immunofluorescence microscope.

10 Positive antibody test results indicate a previous infection with SARS-Coronavirus. Seroconversion from negative to positive or a four-fold rise in antibody titre from acute to convalescent serum indicates recent infection. With negative antibody test results, no detection of antibody after 21 days from onset of illness seems to indicate that no infection with
15 SARS-Coronavirus took place.

Clearly, the current generation of SARS antibody tests, which use the immunofluorescence and ELISA techniques, have a sensitivity problem. Immunofluorescence can only detect the antibodies after a patient has carried the disease for at least
20 10 days, while for ELISA, about 20 days are needed. During that time, the patient may have spread the virus to other people. In that context, the tests are mainly useful after hospitalisation, for confirming whether a patient has SARS. That is the reason why these tests are not very useful for
25 infectious disease control. Thus the need exists for a bedside test, a quick on-the-spot test which allows the detection (from secretions) at an early stage of the disease or the detection of early antibodies, say from day five (of infection) onwards.

Another conventional detection method is constituted by
30 cell cultures. Virus in specimens (such as respiratory secretions, blood or stool) from SARS patients can also be detected by inoculating cell cultures and growing the virus. Once isolated, the virus must be identified as the SARS virus with further tests. Cell culture is a very demanding test, but
35 currently (with the exception of animal trials) the only means

to show the existence of a live virus. Positive cell culture results indicate the presence of live SARS-Coronavirus in the sample tested. Negative cell culture results do not exclude SARS. Moreover these two conventional techniques (detection of antibodies and cell culture) require a laboratory and trained staff as well as time to carry out the test. These tests are good and robust in scientific laboratories but they are not rapid enough to have an efficient diagnosis in due time.

Whereas conventional virus diagnosis has been based predominantly on the detection of viral antigens or specific antibodies thereto, in recent years attention has shifted towards methods for the direct and rapid detection of the genome of viruses or nucleic acid sequences derived thereof, both RNA and DNA. In this respect, the very short time-to-result is a crucial factor to opt for nucleic acid detection. These methods are usually based on nucleic acid hybridization. Nucleic acid hybridization is based on the ability of two strands of nucleic acid containing complementary sequences to anneal to each other under the appropriate conditions, thus forming a double stranded structure. When the complementary strand is labeled, the label can be detected and is indicative for the presence of the target sequence. Especially in combination with methods for the amplification of nucleic acid sequences these methods have become an important tool in viral diagnosis.

Nucleic acid amplification techniques are especially useful as an additional technique in cases where serological methods give doubtful results or in cases where there may be a considerable time period between infection and the development of antibodies to the virus. With HIV for example, seroconversion usually can occur some 3-6 weeks after exposure to the virus. Thus, whereas no antibodies will be detected with conventional immunoassays, proviral DNA or circulating viral RNA may already be detectable. Also in monitoring antiviral

therapy, methods based on nucleic acid amplification have several advantages over serological methods. Especially quantitative amplification methods provide a powerful tool in assessing the changes in the amount of virus present before and during therapy.

The choice of the oligonucleotides to be used as primers and probes in the amplification and detection of nucleic acid sequences is critical for the sensitivity and specificity of the assay. The sequence to be amplified is usually only present in a sample (for example a blood sample obtained from a patient suspected of having a viral infection) in minute amounts. The primers should be sufficiently complementary to the target sequence to allow efficient amplification of the viral nucleic acid present in the sample. If the primers do not anneal properly (due to mispairing of the bases on the nucleotides in both strands) to the target sequence, amplification is seriously hampered. This will affect the sensitivity of the assay and may result in false negative test results. Due to the heterogeneity of viral genomes false negative test results may be obtained if the primers and probes are capable of recognizing sequences present in only part of the variants of the virus.

Various techniques for amplifying nucleic acid are known in the art. One example of a technique for the amplification of a DNA target segment is the so-called "polymerase chain reaction" (PCR). With the PCR technique the copy number of a particular target segment is increased exponentially with a number of cycles. A pair of primers is used and in each cycle a DNA primer is annealed to the 3' side of each of the two strands of the double stranded DNA-target sequence. The primers are extended with a DNA polymerase in the presence of the various mononucleotides to generate double stranded DNA again. The strands of the double stranded DNA are separated from each other by thermal denaturation and each strand serves as a

template for primer annealing and subsequent elongation in a following cycle. The PCR method has been described in Saiki et al., Science 230, 135, 1985 and in patents EP-B-0.200.362 and EP-A-0.201.184.

Another technique for the amplification of nucleic acid is the so-called transcription based amplification system (TAS). The TAS method is described in WO-A-88/10315. Transcription based amplification techniques usually comprise treating target nucleic acid with two oligonucleotides one of which comprises a promoter sequence, to generate a template including a functional promoter. Multiple copies of RNA are transcribed from said template and can serve as a basis for further amplification.

An isothermal continuous transcription based amplification method is the so-called NASBA process ("NASBA") as described in EP-B-0.329.822. NASBA includes the use of T7 RNA polymerase to transcribe multiple copies of RNA from a template including a T7 promoter. Other transcription based amplification techniques are described in EP-A-0.408.295. EP-A-0.408.295 is primarily concerned with a two-enzyme transcription based amplification method. Transcription based amplification methods, such as the NASBA method as described in EP-A-0.329.822, are usually employed with a set of oligonucleotides, one of which is provided with a promoter sequence that is recognized by a DNA dependent RNA polymerase such as, for example, T7 RNA polymerase. Several modifications of transcription-based techniques are known in the art. These modifications comprise, for example, the use of blocked oligonucleotides (that may be provided with a promoter sequence). These oligonucleotides are blocked so as to inhibit an extension reaction proceeding therefrom (US-A-5,554,516). One or more "promoter-primers" (oligonucleotides provided with a promoter sequence) may be used in transcription based amplification techniques,

overlap with the BNI-1 and BNI-2 sequences (Ksiazek et al. (13)).

When primers specifically targeting the BNI-1 fragment and the CDC fragment were used in a long-range PCR protocol, a region extending from the CDC fragment to the BNI-1 fragment was amplified. The obtained fragment had the expected length of 3 kb. It was sequenced from both ends, and the sequences were found to be identical to the CDC and BNI-1 sequences, respectively, demonstrating that the two sequences were derived from a contiguous RNA molecule and, thus, from the same virus. Since the same virus was isolated from two independent cases of SARS, and since there was serologic evidence of an acute infection with this virus in the underlying patients, it was considered that the coronavirus might have a role in causing SARS. Specific diagnostic assays based on RT-PCR were therefore established for the detection of the pathogen.

However, PCR and RT-PCR also have problems. Drosten et al. (10) developed a diagnostic method for SARS that makes use of a nested set of primers designed within the BNI-1 fragment. The outer set of primers detected the virus in clinical specimens from the index patient and different patients, who also had clinical signs of SARS. Additional specimens were positive on nested RT-PCR. To have a practical and quantitative test, a real-time RT-PCR with a 5'-nuclease probe was established. After optimization with the use of quantified RNA transcribed in vitro, the assay reliably detected 10 copies of RNA per reaction, corresponding to 830 RNA molecules per millilitre of specimen. The sensitivities of nested and real-time PCR were equivalent.

The specificity of the PCR with outer primers of the nested PCR, and of the real-time PCR was tested with the use of RNA purified from cultures of bovine coronavirus, avian infectious coronavirus, porcine transmissible gastroenteritis coronavirus and human coronaviruses 229E and OC43. None of the

optionally combined with the use of one or more oligonucleotides that are not provided with a promoter sequence. For RNA amplification, a transcription based amplification technique, is a preferred technology.

Amplification using PCR can also be based on an RNA template. The actual PCR needs to be preceded by a reverse transcription step to copy the RNA into DNA (RT-PCR). However, if RT-PCR is used for the detection of viral transcripts, differentiation of mRNA- and DNA-derived PCR products is necessary. DNase treatment prior to RT-PCR can be employed (Bitsch et al. (8); Meyer et al. (9)), but sometimes fails to remove contaminating DNA sufficiently (Bitsch et al. (8)). That is the solution advocated by Drosten et al. (10) for the detection of the SARS Coronavirus. Thus by performing random amplification with fifteen different PCRs under low-stringency conditions and including, an initial reverse-transcription step to detect RNA viruses, about twenty distinct DNA fragments were obtained and sequenced. Three of the fragments did not match any nucleotide sequence in the database (www.ncbi.nlm.nih.gov:80/BLAST). In a translated BLAST search these fragments showed homology to coronavirus amino acid sequences. Two of the fragments were 300 nucleotides in length and identical in sequence, and the third fragment was 90 nucleotides in length (sequences BNI-1 and BNI-2, respectively, as reported on the Web site of the WHO network on March 25). Detailed sequence analysis revealed that both fragments were located in the open reading frame 1b of coronaviruses. The novel coronavirus isolate was termed FFM-ic (for Frankfurt am Main index case).

Simultaneously, a group at the Centers for Disease Control and Prevention (CDC), Atlanta, USA identified a 400-nucleotide coronavirus fragment (Urbani isolate, reported on the Web site of the WHO network on March 24). This sequence did not show an

PCR assays cross-reacted with these viruses - a finding that is consistent with their considerable genetic differences from the novel coronavirus. Quantification of the viral RNA

5 concentration in clinical specimens from the index patient and Contact 1 by real-time PCR revealed that the highest concentration - as high as 100 million copies per millilitre - was present in sputum. After enrichment of virus by ultra centrifugation, viral RNA was also detected in the serum of the
10 index patient, indicating the presence of low-level viremia during symptomatic disease. Both the index patient and Contact 1 had viral RNA in stool samples obtained during late convalescence, suggesting that virus may be shed in faeces for prolonged periods of time. To investigate whether the novel
15 coronavirus was prevalent in patients in Germany who had gastrointestinal symptoms, a collection of fifty-four stored stool samples was tested with the use of the real-time RT-PCR assay. None of the samples tested positive.

The established PCR assays have also been used to test
20 respiratory samples from German patients with symptoms and a travel history compatible with SARS. So far, sixty-seven samples from fifty-five patients have been tested. One patient fulfilling the WHO criteria for probable SARS was coronavirus-positive on PCR. PCR protocols, as well as positive control
25 material, have been made available to laboratories worldwide. Until standardized reagents for virus and antibody detection are available and methods have been adequately field tested, SARS diagnosis remains based on the clinical and
30 epidemiological findings: acute febrile illness with respiratory symptoms not attributed to another cause and a history of exposure to a suspect or probable case of SARS or their respiratory secretions and other bodily fluids. Those requirements are reflected in the current WHO case definitions for suspect or probable SARS.

Researchers in several countries are working towards developing fast and accurate laboratory diagnostic tests for the SARS-Coronavirus. Molecular tests based on PCR
5 amplification are one of the faster and more accurate tests that could achieve this goal.

Polymerase chain reaction (PCR) can detect genetic material of the SARS-Coronavirus in various specimens (blood, stool, respiratory secretions or body tissues). Same primers,
10 which are the key pieces for a PCR test, have been made publicly available by WHO network laboratories on the WHO web site. A ready-to-use PCR test kit containing primers and a positive and negative control has been developed. Testing of the kit by network members is expected to quickly yield the
15 data needed to assess the test's performance, in comparison with primers developed by other WHO network laboratories and in correlation with clinical and epidemiological data.

Principally, existing PCR tests are very specific but lack sensitivity. This means that negative tests cannot rule out the
20 presence of the SARS virus in patients. Furthermore, contamination of samples in laboratories in the absence of laboratory quality control can lead to false positive results. Positive PCR results, with the necessary quality control procedures in place, are very specific and mean that there is
25 genetic material (RNA) of the SARS-Coronavirus in the sample. This does not mean that there is live virus present, or that it is present in a quantity large enough to infect another person. Negative PCR results do not exclude SARS. SARS-Coronavirus PCR can be negative for the following reasons:

- 30 - the patient is not infected with the SARS coronavirus; the illness is due to another infectious agent (virus, bacterium, fungus) or a non-infectious cause,
- the test results are incorrect ("false-negative"). Current tests need to be further developed to improve sensitivity,
- 35 - specimens were not collected at a time when the virus or its

genetic material was present. The virus and its genetic material may be present for a brief period only, depending on the type of specimen tested,

- 5 - specimens were not properly handled prior to nucleic acid extraction and nucleic acids have become deteriorated.

It is the purpose of this invention to provide reagents and methods to realise kits for diagnosing SARS disease earlier than former kits. The use of the oligonucleotides according to the invention is not limited to any particular amplification technique or any particular modification thereof, however these oligonucleotides permit amplification of RNA by using a transcription-based amplification technique, preferably the NASBA. Our primers sets are very efficient compared to the RT-PCR primers. In contrast to RT-PCR, NASBA, which is based on RNA transcription by T7 RNA polymerase (Kievits et al. (11); Compton (12)), does not need differentiation between RNA- and DNA-derived amplification products since it uses RNA as its principal target. RT-PCR for SARS have a too low sensitivity, often nested PCR protocol needed to achieve satisfying sensitivity. The nested protocol is then riskful in terms of contamination, i.e. false positives. NASBA can achieve sensitivity comparable to nested protocol in a "one-step" amplification avoiding risk for contamination.

25 The oligonucleotides of the present invention can likewise be used in quantitative amplification methods. An example of such quantitative method is described in EP-A-0.525.882. The term "oligonucleotide" as used herein refers to a molecule comprised of two or more deoxyribonucleotides or
30 ribonucleotides. Such oligonucleotides may be used as primers and probes.

Of course, based on the sequences of the oligonucleotides of the present invention, analogues of oligonucleotides can also be prepared. Such analogues may constitute alternative
35 structures such as "PNA" (molecules with a peptide-like

backbone instead of the phosphate sugar backbone of normal nucleic acid) or the like. It is evident that these alternative structures, representing the sequences of the present invention
5 are likewise part of the present invention.

The term "primer" as used herein refers to an oligonucleotide either naturally occurring (e.g. as a restriction fragment) or produced synthetically, which is capable of acting as a point of initiation of synthesis of a
10 primer extension product which is complementary to a nucleic acid strand (template or target sequence) when placed under suitable conditions (e.g. buffer, salt, temperature and pH) in the presence of nucleotides and an agent for nucleic acid polymerization, such as DNA dependent or RNA dependent
15 polymerase. A primer must be sufficiently long to prime the synthesis of extension products in the presence of an agent for polymerization. A typical primer contains at least about 10 nucleotides in length of a sequence substantially complementary or homologous to the target sequence, but somewhat longer
20 primers are preferred. Usually primers contain about 15-28 nucleotides, preferably 20-26 nucleotides, but longer primers may also be employed, especially when the primers contain additional sequences such as a promoter sequence for a particular polymerase.

25 Normally a set of primers will consist of at least two primers, one "upstream" primer and one "downstream" primer, which together define the amplicon (the sequence that will be amplified using said primers).

Primarily for the use in transcription based amplification
30 techniques, the oligonucleotides according to the invention may also be linked to a promoter sequence. The term "promoter sequence" defines a region of a nucleic acid sequence that is specifically recognized by an RNA polymerase that binds to a recognized sequence and initiates the process of transcription
35 by which an RNA transcript is produced. In principle any

promoter sequence may be employed for which there is a known and available polymerase that is capable of recognizing the initiation sequence. Known and useful promoters are those that are recognized by certain bacteriophage RNA polymerases such as bacteriophage T3, T7 or SP6. Oligonucleotides linked to a promoter sequence are commonly referred to as "promoter primers". Their function as a primer, e.g. the starting point for an elongation reaction, however, may be blocked, as already mentioned above, or absent in some embodiments of transcription based amplification reactions.

It is understood that oligonucleotides consisting of the sequences of the present invention may contain minor deletions, additions and/or substitutions of nucleic acid bases, to the extent that such alterations do not negatively affect the yield or product obtained to a significant degree. Where oligonucleotides according to the present invention are used as probes, the alterations should not result in lowering the hybridization efficiency of the probe. For example, in case of transcription based amplification techniques, wherein one or more of the primers may be provided with a promoter sequence, the introduction of a purine-rich (= G or A) hybridizing sequence, just after the promoter sequence may have positive effects on the transcription (when there are C's and T's abortive transcription may occur). If no such sequence is available in the target nucleic acid a purine-rich sequence can be inserted in the oligonucleotide just following the last three G residues of the promoter sequence.

The sequences of the present invention are reflected as DNA sequences. The RNA equivalents of these sequences are likewise part of the present invention.

An aspect of the invention is a pair of oligonucleotides, for use as a set in the amplification of a target sequence of the genome of SARS Coronavirus, said pair consisting of:

a first oligonucleotide being 10-50 nucleotides in length and comprising at least a fragment of 10 nucleotides of:

SEQ ID 1: TACCTCTCCA GCTAGGATTT TCTACAGGTG TTAAGTTAGT
AGCTGTACCG ACTGGTTATG TTGACACTGA AAATAACACA GAATTCACCA
5 GAGTTAATGC AAAACCTCCA CCAGGTGACC AGTTTAAACA TCTT,

SEQ ID 14: TCAGCCCCAG ATGGTACTTC TATTACCTAG GAACTGGCCC
AGAAGCTTCA CTT,

SEQ ID 23: TGCTCCAAGT GCCTCTGCAT TCTTTGGAAT GTCACGCATT
GGCATGGAAG TCACACCTT, or

10 SEQ ID 31: TGCCTATATG GAAGAGCCCT AATGTGTAAA ATTAATTTTA
GTAGTGCTAT CCCCATGTGA TTTAATAGC TT,

or the complementary sequence thereof,

a second oligonucleotide being 10-50 nucleotides in length and comprising at least a fragment of 10 nucleotides of:

15 SEQ ID 2: ATGAATTACC AAGTCAATGG TTACCCTAAT ATGTTTATCA
CCGCGAAGA AGCTATTCGT CACGTTTCGTG CGTGGATTGG CTTTGATGT,

SEQ ID 17: AGGTTTACCC AATAATACTG CGTCTTGGTT CACAGCTCTC
ACTCAGCATG GCAAGGAGGA ACTTAGATTC CCTCGAGGCC AGGGCGTTCC
AATCAACACC AATAGTGGTC CAGATGACCA AAT,

20 SEQ ID 26: CCAAAGTGC ACTAAGAAAT CTGCTGCTGA GGCATCTAAA
AAGCCTCGCC AAAAACGTAC TGCCACAAAA CAGTACAACG TCACTCAAGC
ATTTGGGAGA CGTGGTCCAG AACAAACCCA AGGAAATT, or

SEQ ID 34: TACGATACAT AGTCTACTCT TGTGCAGAAT GAATTCTCGT
AACTAAACAG CACAAGTAGG TTTAGTTAAC TTTAATCTCA CATAGCAATC
25 TTTAATCAAT GT,

or the complementary sequence thereof.

More specifically, the pair of oligonucleotides, according to the above definition, consists essentially of:

a first oligonucleotide comprising, at least a fragment of
30 10 nucleotides, of a sequence selected from the group
consisting of:

SEQ ID 3: TCCACCAGGT GACCAGTTTA AACATCTT,

SEQ ID 4: TAGTAGCTGT ACCGACTGGT TATGTT,

SEQ ID 5: TACCTCTCCA GCTAGGATTT TCT,

35 SEQ ID 15: TCAGCCCCAG ATGGTACTTC T,

SEQ ID 16: TAGGAACTGG CCCAGAAGCT TCACTT,

SEQ ID 24: TGCTCCAAGT GCCTCTGCAT TCTT,

SEQ ID 25: TTGGCATGGA AGTCACACCT T,

5 SEQ ID 32: TGCCTATATG GAAGAGCCC,

SEQ ID 33: TCCCCATGTG ATTTTAATAG CTT,

or the complementary sequence thereof, and

a second oligonucleotide comprising, at least a fragment of 10 nucleotides, of a sequence selected from the group

10 consisting of:

SEQ ID 6: ATGAATTACC AAGTCAATGG TTAC,

SEQ ID 7: GAAGCTATTC GTCACGTTTCG,

SEQ ID 8: TCGGTGGATT GGCTTTGATG T,

SEQ ID 18: AGGTTTACCC AATAATACTG CGT,

15 SEQ ID 19: AGATTCCCTC GAGGCCAGGG CGT,

SEQ ID 20: ATAGTGGTCC AGATGACCAA AT,

SEQ ID 27: CCAAAGTGC ACTAAGAAAT CTGCT,

SEQ ID 28: CTCAAGCATT TGGGAGACGT GGT,

SEQ ID 29: CAGAACAAC CCAAGGAAAT T,

20 SEQ ID 35: TACGATACAT AGTCTACTCT TGT,

SEQ ID 36: TAACTAAACA GCACAAGTAG GT,

SEQ ID 37: TAGCAATCTT TAATCAATGT,

or the complementary sequence thereof.

Another aspect of the invention is a pair of
25 oligonucleotides, for use as a set in the amplification of a target sequence located within the replicase gene of the genome of SARS Coronavirus, said pair consisting of:

a first oligonucleotide being 10-50 nucleotides in length and comprising at least a fragment of 10 nucleotides of: SEQ ID
30 1: TACCTCTCCA GCTAGGATTT TCTACAGGTG TTAAGTTAGT AGCTGTACCG
ACTGGTTATG TTGACACTGA AAATAACACA GAATTCACCA GAGTTAATGC
AAAACCTCCA CCAGGTGACC AGTTTAAACA TCTT, or the complementary sequence thereof, and

a second oligonucleotide being 10-50 nucleotides in length
35 and comprising at least a fragment of 10 nucleotides of:

SEQ ID 2: ATGAATTACC AAGTCAATGG TTACCCTAAT ATGTTTATCA
 CCCGCGAAGA AGCTATTCGT CACGTTTCGTG CGTGGATTGG CTTTGATGT, or the
 complementary sequence thereof.

5 More specifically, the pair of oligonucleotides, according
 to the above definition, consists essentially of:

a first oligonucleotide comprising at least a fragment of
 10 nucleotides of a sequence selected from the group consisting
 of:

10 SEQ ID 3: TCCACCAGGT GACCAGTTTA AACATCTT,

SEQ ID 4: TAGTAGCTGT ACCGACTGGT TATGTT,

SEQ ID 5: TACCTCTCCA GCTAGGATTT TCT,

or the complementary sequence thereof, and

a second oligonucleotide comprising at least a fragment of
 15 10 nucleotides of a sequence selected from the group consisting
 of:

SEQ ID 6: ATGAATTACC AAGTCAATGG TTAC,

SEQ ID 7: GAAGCTATTC GTCACGTTTCG,

SEQ ID 8: TCGTGATT GGCTTTGATG T,

20 or the complementary sequence thereof.

Another aspect of the invention is a pair of
 oligonucleotides, for use as a set in the amplification of a
 target sequence located within the gene encoding the
 Nucleocapsid protein of the genome of SARS Coronavirus, said
 25 pair consisting of:

a first oligonucleotide being 10-50 nucleotides in length
 and comprising at least a fragment of 10 nucleotides of:

SEQ ID 14: TCAGCCCCAG ATGGTACTTC TATTACCTAG GAACTGGCCC
 AGAAGCTTCA CTT, or the complementary sequence thereof, or

30 a first oligonucleotide being 10-50 nucleotides in length
 and comprising at least a fragment of 10 nucleotides of: SEQ ID
 23: TGCTCCAAGT GCCTCTGCAT TCTTTGGAAT GTCACGCATT GGCATGGAAG
 TCACACCTT, or the complementary sequence thereof, and

a second oligonucleotide being 10-50 nucleotides in length
 35 and comprising at least a fragment of 10 nucleotides of:

SEQ ID 17: AGGTTTACCC AATAATACTG CGTCTTGGTT CACAGCTCTC
 ACTCAGCATG GCAAGGAGGA ACTTAGATTC CCTCGAGGCC AGGGCGTTCC
 AATCAACACC AATAGTGGTC CAGATGACCA AAT, or the complementary
 5 sequence thereof or,

a second oligonucleotide being 10-50 nucleotides in length
 and comprising at least a fragment of 10 nucleotides of:

SEQ ID 26: CCAAAGTGTG ACTAAGAAAT CTGCTGCTGA GGCATCTAAA
 AAGCCTCGCC AAAAACGTAC TGCCACAAAA CAGTACAACG TCACTCAAGC
 10 ATTTGGGAGA CGTGGTCCAG AACAAACCCA AGGAAATT, or the complementary
 sequence thereof.

More specifically, the pair of oligonucleotides, according
 to the above definition, consists essentially of:

a first oligonucleotide comprising at least a fragment of
 15 10 nucleotides of a sequence selected from the group consisting
 of:

SEQ ID 15: TCAGCCCCAG ATGGTACTTC T,
 SEQ ID 16: TAGGAAGTGG CCCAGAAGCT TCACTT,
 SEQ ID 24: TGCTCCAA GTGCCTCTGC ATTCTT,
 20 SEQ ID 25: TTGGCATGGA AGTCACACCT T,
 or the complementary sequence thereof, and

a second oligonucleotide comprising at least a fragment of
 10 nucleotides of a sequence selected from the group consisting
 of:

25 SEQ ID 18: AGGTTTACCC AATAATACTG CGT,
 SED ID 19: AGATTCCCTC GAGGCCAGGG CGT,
 SEQ ID 20: ATAGTGGTCC AGATGACCAA AT,
 SEQ ID 27: CCAAAGTGTG ACTAAGAAAT CTGCT,
 SED ID 28: CTCAAGCATT TGGGAGACGT GGT,
 30 SEQ ID 29: CAGAACAAAC CCAAGGAAAT T,
 or the complementary sequence thereof.

Another aspect of the invention is a pair of
 oligonucleotides, for use as a set in the amplification of a
 target sequence located within the 3'-Non Coding Region (3'-
 35

NCR) of the genome of SARS Coronavirus, said pair consisting of:

a first oligonucleotide being 10-50 nucleotides in length and comprising at least a fragment of 10 nucleotides of: SEQ ID
 5 31: TGCCTATATG GAAGAGCCCT AATGTGTAAA ATTAATTTTA GTAGTGCTAT
 CCCCATGTGA TTTTAATAGC TT, or the complementary sequence thereof, and

a second oligonucleotide being 10-50 nucleotides in length and comprising at least a fragment of 10 nucleotides of: SEQ ID
 10 34: TACGATACAT AGTCTACTCT TGTGCAGAAT GAATTCTCGT AACTAAACAG
 CACAAGTAGG TTTAGTTAAC TTTAATCTCA CATAGCAATC TTTAATCAAT GT, or the complementary sequence thereof.

More specifically, the pair of oligonucleotides, according to the above definition, consists essentially of:

15 a first oligonucleotide comprising at least a fragment of 10 nucleotides of a sequence selected from the group consisting of:

SEQ ID 32: TGCCTATATG GAAGAGCCC,

SEQ ID 33: TCCCATGTG ATTTTAATAG CTT,

20 or the complementary sequence thereof, and

a second oligonucleotide comprising at least a fragment of 10 nucleotides of a sequence selected from the group consisting of:

SEQ ID 35: TACGATACAT AGTCTACTCT TGT,

25 SED ID 36: TAACTAAACA GCACAAGTAG GT,

SEQ ID 37: TAGCAATCTT TAATCAATGT,

or the complementary sequence thereof.

The oligonucleotide pair, according to any of the above definitions, may be constituted by a first oligonucleotide
 30 provided with a promoter sequence recognized by a DNA dependent RNA polymerase.

For instance, the sequences SEQ ID 9-11 and SEQ ID 39-44 actually comprise the complementary and reverse sequence as reflected by SEQ ID 3-5 and SEQ ID 15, 16, 24, 25, 32 and 33

respectively. In SEQ ID 9-11 and SEQ ID 39-44, the complementary and reverse sequences of SEQ ID 3-5 and SEQ ID 15, 16, 24, 25, 32 and 33 are operably linked to a promoter sequence (the T7 promoter sequence). This makes the sequences especially suitable for use as downstream primer in a transcription based amplification technique such as NASBA.

One of the oligonucleotides may serve as an "upstream oligonucleotide", i.e., an upstream-primer, while the second oligonucleotide serves as a "downstream oligonucleotide", i.e. downstream primer, in the amplification reaction. The location on the SARS-genome (or the sequence complementary thereto) to which both oligonucleotides comprised in such a pair according to the invention can anneal, will together define the sequence of the nucleic acid that is amplified. The amplified sequence is located between the "primer-binding sites" within the SARS genome. It has been found that, by using a pair of oligonucleotides according to the invention in an amplification reaction, accurate and reliable amplification of nucleic acid of SARS can be achieved.

A preferred pair of oligonucleotides according to the invention in the SARS-CoV REP consist of a first primer comprising the sequence of SEQ ID NO 4 or 10 and a second primer with the sequence of SEQ ID NO 7, or of a second set composed by a first primer comprising the sequence of SEQ ID NO 10 and by a second primer with the sequence of SEQ ID NO 8, or of a third set composed by a first primer comprising the sequence of SEQ ID NO 9 and by a second primer with the sequence of SEQ ID NO 8. For use in a transcription based amplification method, especially the NASBA amplification, the oligonucleotide with SEQ ID NO 10 is preferred, in combination with an oligonucleotide with the sequence of SEQ ID NO 7.

A most preferred pair of oligonucleotides according to the invention in the SARS-CoV N:

in a first part of the nucleic acid sequence, hereafter called region 1, consists of a first primer comprising the sequence of SEQ ID NO 15 and a second primer with the sequence of SEQ ID NO 19, and

in a second part of the nucleic acid sequence, hereafter called region 2, consists of either

a first primer comprising the sequence of SEQ ID NO 41 and a second primer with the sequence of SEQ ID NO 28, or

a first primer comprising the sequence of SEQ ID NO 42 and a second primer with the sequence of SEQ ID NO 28, or

a first primer comprising the sequence of SEQ ID NO 42 and a second primer with the sequence of SEQ ID NO 29.

A most preferred pair of oligonucleotides according to the invention in the SARS-CoV 3'-NCR consist of a first set composed by a first primer comprising the sequence of SEQ ID NO 32 and by a second primer with the sequence of SEQ ID NO 35 and a second set composed by a first primer comprising the sequence of SEQ ID NO 33 and by a second primer with the sequence of SEQ ID NO 36.

Part of the oligonucleotides according to the invention are particularly suitable for use as a probe in the detection of nucleic acid amplified with a pair of oligonucleotides according to the invention. When used as a probe, said oligonucleotides may be provided with a detectable label.

Oligonucleotides according to the invention that are especially suitable as a probe consist of sequences of 10-50 nucleotides in length and comprising at least a fragment of 10 nucleotides of:

SEQ ID 12: GTTCGTGCGT GGATTGGCTT TGATGTAGAG GGCTGTCATG
CAACTAGAGA TGCTGT,

SEQ ID 21: GGCTACTACC GAAGAGCTAC CCGACGAGTT CGTGGTGGTG
 ACGGCAAAAT GAAAGAGCTC AGCCCCAGAT GGTACTTCTA TTACCTAGGA
 ACTGGCCCAG AAGCTTCACT TCCCTACGGC GCTAACAAAG AAGGCATCGT
 5 ATGGGTGCA ACTGAGGGAG CCTTGAATAC ACCCAAAGAC CACATTGGCA
 CCCGCAATCC TAATAACAAT GCTGCCACCG TGCTACAACT TCCTCAAGGA
 ACAACATTGC CAAAAGGCTT CTACGCAGAG GGAAGCAGAG GCGGCAGTCA
 AGCCTCTTCT CGCTCCTCAT CACGTAGTCG CGGTAATTCA AGAAATTCAA
 CTCCTGGCAG CAGTAGGGGA AATTCTCCTG CTCGAATGGC TAGCGGAGGT
 10 GGTGAAACTG CCCTCGCGCT ATTGCTGCTA GACAGATTGA ACCAGCTTGA
 GAGCAAAGTT TCTGGTAAAG GCCAACAACA ACAAGGCCAA ACTGTCATA
 AGAAATCTGC TGCTGAGGCA TCTAAAAAGC CTCGCCAAAA ACGTACTGCC
 ACAAACAGT ACAACGTCAC TCAAGCATTT GGGAGACGTG GTCCAGAACA
 AACCAAGGA AATTCGGGG ACCAAGACCT AATCAGACAA,
 15 SEQ ID 38: GCCACCACAT TTTCATCGAG GC,
 or the complementary sequence thereof, provided with a
 detectable label.

A molecular beacon, preferably consisting of, constitutes
 the probe:

20 SEQ ID 13: 5'-[6-FAM]-ccatgggCTGTCATGCAACTAGAGATGCTGTcccatgg-
 [DabSyl]-3',
 SEQ ID 45: 5'-[6-FAM]-cgcgatGTTCGTGCGTGATTGGCTTatcgcg-
 [DabCyl]-3',
 SEQ ID 22: 5'-[6-FAM]-ccatgggCTACTACCGAAGAGCTACCCGACGAcccatgg-
 25 [DabSyl]-3',
 SEQ ID 30: 5'-[6-FAM]-ccatggACCAAGACCTAATCAGACAaccatgg-
 [DabSyl]-3',
 SEQ ID 47: 5'-[6-FAM]-ccatgcGCCACCACATTTTCATCGAgcatgg-[DabSyl]-
 3'.

30 Various labeling moieties are known in the art. Said
 moiety may, for example, either be a radioactive compound, a
 detectable enzyme (e.g. horse radish peroxidase (HRP)), a
 hapten like biotin, or any other moiety capable of generating a
 detectable signal such as a colorimetric, fluorescent,
 35 chemiluminescent or electrochemiluminescent signal.

Hybrids between oligonucleotides according to the invention and (amplified) target nucleic acid may also be detected by other methods known to those skilled in the art.

5 Evidently methods for amplification of nucleic acid, like the ones that have been mentioned above, using the oligonucleotides according to the present invention are also part of the invention.

10 The sequence in capital letters refers to the probe itself according to SEQ ID 14 for instance, i.e. where the hybridisation between said probe and a part of the amplified nucleic acid takes place. Downstream and upstream extensions, in small letters, are sequences called here 7-nucleotide-long arm sequences that are able to hybridise one to the other.

15 These arm sequences relate to linkers between the probe and on the one hand a fluorophore (6-FAM) and on the other hand quencher 4-(4'-dimethylaminophenylazo) benzoic acid (DabCyl or DabSyl). An ideal fluorophore-quencher pair is completely unable to fluoresce when the two components are in close

20 proximity, but as soon as the loop of the molecular beacon is conformationally changed due to hybridization of the probe to the amplified nucleic acid, the distance between the fluorophore and the quencher is sufficiently enlarged to permit emission of fluorescence.

25 Other fluorescent reporters are possible like 5' fluorescein, 5' TET, 5' HEX, 5' FAM, 5' TAMRA, 5' ROX, 5' Texas Red, 5' Oregon Green, 5' Cy3 or 5' Cy5. In the same way other quenchers, such as 3' Black Hole Quencher-1 or 3' Black Hole Quencher-2, can be used.

30 In another aspect of the invention, an oligonucleotides' pair, according to the ones here above disclosed, is used in a nucleic acid amplification reaction or as a probe for the detection of SARS Coronavirus nucleic acid in a sample. Consequently it is possible to perform a method for the

35 detection of SARS nucleic acid in a sample wherein the sample

is subjected to a nucleic acid amplification reaction using a pair of oligonucleotides above disclosed and suitable amplification reagents and the presence of any amplified nucleic acid is detected. This method, wherein reacting the sample with an oligonucleotide under suitable hybridization conditions and detecting the presence of the label in any hybrids could carry out the detection of any amplified nucleic acid, formed between the amplified sequence and the probe. Preferably the amplification technique used is a transcription based amplification technique, more preferably the NASBA, and the first oligonucleotide is provided with a promoter sequence recognized by a DNA dependent RNA polymerase.

The present invention further provides test kits for the amplification and detection of SARS nucleic acid. The use of said test kits enables accurate and sensitive screening of samples suspected of containing SARS derived nucleic acid. Such test kits may contain a pair of oligonucleotides according to the invention and optionally also an oligonucleotide according to the invention that can be used as a probe for the detection of the amplified material. Furthermore the test kit may contain suitable amplification reagents. These reagents are for example the suitable enzymes for carrying out the amplification reaction. A kit, adapted for use with NASBA, for example may contain suitable amounts of reverse transcriptase, RNase H and T7 RNA polymerase. Said enzymes may be present in the kit in a buffered solution but can likewise be provided as a lyophilized composition, for example, a lyophilized spherical particle. Such lyophilized particles have been disclosed in PCT/EP95/01268. The kit may further be furnished with buffer compositions, suitable for carrying out an amplification reaction. Said buffers may be optimized for the particular amplification technique for which the kit is intended as well as for use with the particular oligonucleotides that are provided with the kit. In transcription-based amplification

techniques, such as NASBA, said buffers might contain, for example, DMSO, which enhances the amplification reaction (as is disclosed in PCT/US90/04733).

5 Furthermore the kit may be provided with an internal control as a check on the amplification procedure and to prevent the occurrence of false negative test results due to failures in the amplification procedure. The use of internal controls in transcription based amplification techniques is described in PCT/EP93/02248. An optimal control sequence is selected in such a way that it will not compete with the target nucleic acid in the amplification reaction. Kits may also contain reagents for the isolation of nucleic acid from biological specimens prior to amplification. A suitable method for the isolation of nucleic acid is disclosed in EP-A-0.389.063.

Virus propagation and isolation from in-vitro cultures:

A variety of clinical specimens (blood, serum, material from oropharyngeal swabs or washings, material from nasopharyngeal swabs, and tissues of major organs collected at autopsy) were inoculated onto a number of continuous cell lines, including Vero E6, NCIH292, MDCK, LLC-MK2 and B95-8 cells. Two cell lines, Vero E6 cells and NCI-H292 cells, inoculated with oropharyngeal specimens from patients with SARS initially showed cytopathic effect. A rhinovirus was isolated from the inoculated NCI-H292 cells, but further study suggested that this virus was not associated with patients with SARS. Examination of cytopathic-effect-positive VeroE6 cells by thin-section electron microscopy revealed characteristic coronavirus particles within the cisternae of the rough endoplasmic reticulum and in vesicles. Extracellular particles were found in large clusters and adhering to the surface of the plasma membrane. Vero E6 cells have now become the standard cell line for the isolation and propagation of the SARS related human

Coronavirus (Marra et al. (1), Drosten et al. (10), Ksiazek et al. (13)).

Identification of specific nucleic acid fragments of the SARS related human Coronavirus:

This identification has already been disclosed previously in the disclosure according to a publication (Drosten et al. (10)). A group at the National Microbiology Laboratory in Canada also obtained a SARS genomic sequence (Genbank Accession AY274119.3) from an isolate referred to as Tor2. The latter sequence is essentially identical to the one that was released independently by the CDC (GenBank accession number AY278741). Additional bases in the Tor2 sequence that correspond to the 3' (encoded) polyA tail were reported. Eight base differences between the two sequences could represent sequencing errors, PCR artefacts or mutable sites in the genome. (Marra et al. (6)). The genome of SARS-Coronavirus is a 29,727-nucleotides polyadenylated RNA, and 41% of the residues are G or C. The genomic organization is typical of coronaviruses, with the characteristic gene order. The SARS-Coronavirus replicase gene, which comprises approximately two-thirds of the genome, is predicted to encode two polyproteins (encoded by ORF1a and ORF1b) that undergo co-translational proteolytic processing. There are four open reading frames (ORFs) downstream of replicase that are predicted to encode the structural proteins S, E, M, and N, which are common to all known coronaviruses. The hemagglutinin-esterase (HE) gene, which is present between ORF1b and S in group 2 and some group 3 coronaviruses, was not found. (Rota et al. (7)). This is in agreement with the Electron microscopic observations of virus particles lacking the HE surface projections.

Phylogenetic analyses of the sequence of SARS-Coronavirus indicate that SARS-Coronavirus is not closely related to any of the previously characterized coronaviruses and forms a distinct group within the genus Coronavirus. The SARS-Coronavirus is

approximately equidistant from all previously characterized coronaviruses, just as the existing groups are from one another. No evidence for recombination was detected when the predicted protein sequences were analyzed using SimPlot in comparison to sequences of known Coronaviruses.

The coronavirus subgenomic mRNAs are synthesized through a discontinuous transcription process, the mechanism of which has not been unequivocally established. The favoured model for production of subgenomic mRNAs of coronaviruses proposes that discontinuous transcription occurs during synthesis of the negative strand (Sawicki G. S. et al. (14)). The positions of the transcription regulating sequences (TRS) in the genome of SARS-Coronavirus predict that subgenomic mRNAs of 8.3, 4.5, 3.4, 2.5, 2.0, and 1.7 kb, not including the poly(A) tail, should be produced.

At least five subgenomic mRNAs were indeed detected by Northern hybridization of RNA from SARS-Coronavirus-infected cells, using a probe derived from the 3'-untranslated region. Full-length genomic RNA was not detected, likely because it is the least prevalent viral RNA in infected cells (Lai et al. (15)). The predicted 2.0 kb transcript was also not detected, suggesting that the consensus TRS at nucleotides 27,771-27,778 is not used or that it is a low-abundance mRNA (Rota et al. (2)).

The invention will further illustrated by the following non-limiting Examples, with reference to the accompanying Figures, in which:

Figures 1-4 show amplification curves of 4 different primer pairs ((Figure 1) SARS-CoV REP P1.1/P2.1, (Figure 2) SARS-CoV REP P1.1/P2.2, (Figure 3) SARS-CoV REP P1.2/P2.1 and (Figure 4) SARS-CoV REP P1.2/P2.2) tested on a dilution series of SARS Coronavirus *in vitro* REP RNA.

Figure 5 shows amplification curves of the selected primer pair (P1.1/P2.2), testing the analytical sensitivity of the SARS CoV NASBA.

Figure 6 shows the predicted secondary structures for SARS-CoV REP MB-1 and SARS-CoV REP MB-2.

Figures 7-11 show amplification curves of 5 other different primer pairs ((Figure 7) SARS-CoV REP P1.1/P2.3, (Figure 8) SARS-CoV REP P1.2/P2.3, (Figure 9) SARS-CoV REP P1.3/P2.1, (Figure 10) SARS-CoV REP P1.3/P2.2 and (Figure 11) SARS-CoV REP P1.3/P2.3) tested on a dilution series of SARS Coronavirus RNA.

Figure 12 show the predicted secondary structures for SARS-CoV N MB-1 and SARS-CoV N MB-2.

Figures 13-16 show real-time amplification of SARS-CoV viral RNA with SARS-CoV N primer/beacon mixtures in region 1. Four SARS-CoV N primer pairs (Table 7) were combined with molecular beacon SARS-CoV N MB-1 (Table 6) for the amplification and real-time detection of SARS-CoV RNA extracted from a dilution series of cultured SARS-CoV. (Figure 13) SARS-CoV N P1.1/P2.1; (Figure 14) SARS-CoV N P1.1/P2.2; (Figure 15) SARS-CoV N P1.2/P2.1; (Figure 16) SARS-CoV N P1.2/P2.2.

Figures 17-20 show real-time amplification of SARS-CoV viral RNA with SARS-CoV N primer/beacon mixtures in region 1. Four SARS-CoV N primer pairs (Table 7) were combined with molecular beacon SARS-CoV N MB-1 (Table 6) for the amplification and real-time detection of SARS-CoV RNA extracted from a dilution series of cultured SARS-CoV upon storage of the nucleic acid extracts prior to amplification. (Figure 17) SARS-CoV N P1.1/P2.1; (Figure 18) SARS-CoV N P1.1/P2.2; (Figure 19) SARS-CoV N P1.2/P2.1; (Figure 20) SARS-CoV N P1.2/P2.2.

Figures 21-24 show real-time amplification of SARS-CoV viral RNA with SARS-CoV N primer/beacon mixtures in region 2. Four SARS-CoV N primer pairs (Table 7) were combined with molecular beacon SARS-CoV N MB-2 (Table 6) for the amplification and real-time detection of SARS-CoV RNA extracted

from a dilution series of cultured SARS-CoV. (Figure 21) SARS-CoV N P1.3/P2.3; (Figure 22) SARS-CoV N P1.3/P2.4; (Figure 23) SARS-CoV N P1.4/P2.3; (Figure 24) SARS-CoV N P1.4/P2.4.

5 Figures 25-28 show real-time amplification of SARS-CoV viral RNA with SARS-CoV N primer/beacon mixtures in region 2. Four SARS-CoV N primer pairs (Table 6) were combined with molecular beacon SARS-CoV N MB-2 (Table 5) for the amplification and real-time detection of SARS-CoV RNA extracted
10 from a dilution series of cultured SARS-CoV upon storage of the nucleic acid extracts prior to amplification. (Figure 25) SARS-CoV N P1.3/P2.3; (Figure 26) SARS-CoV N P1.3/P2.4; (Figure 27) SARS-CoV N P1.4/P2.3; (Figure 28) SARS-CoV N P1.4/P2.4.

15 Figures 29-34 show real-time amplification of SARS-CoV viral RNA with SARS-CoV N primer/beacon mixtures in region 1. Six SARS-CoV N primer pairs (Table 7) were combined with molecular beacon SARS-CoV N MB-1 (Table 6) for the amplification and real-time detection of SARS-CoV RNA extracted
20 from a dilution series of cultured SARS-CoV. (Figure 29) SARS-CoV N P1.1/P2.1; (Figure 30) SARS-CoV N P1.1/P2.2; (Figure 31) SARS-CoV N P1.1/P2.5; (Figure 32) SARS-CoV N P1.2/P2.1; (Figure 33) SARS-CoV N P1.2/P2.2; (Figure 34) SARS-CoV N P1.2/P2.5.

25 Figures 35-40 show real-time amplification of SARS-CoV viral RNA with SARS-CoV N primer/beacon mixtures in region 2. Six SARS-CoV N primer pairs (Table 7) were combined with molecular beacon SARS-CoV N MB-2 (Table 6) for the amplification and real-time detection of SARS-CoV RNA extracted
30 from a dilution series of cultured SARS-CoV. (Figure 35) SARS-CoV N P1.3/P2.3; (Figure 36) SARS-CoV N P1.3/P2.4; (Figure 37) SARS-CoV N P1.3/P2.6; (Figure 38) SARS-CoV N P1.4/P2.3; (Figure 39) SARS-CoV N P1.4/P2.4; (Figure 40) SARS-CoV N P1.4/P2.6.

Figure 41 show predicted secondary structures for the SARS-CoV 3'-NCR molecular beacon.

35 Figure 42-47 show real-time amplification of SARS-CoV viral RNA with SARS-CoV 3'-NCR primer/beacon mixtures. Six

SARS-CoV 3'-NCR primer pairs (Table 9) were combined with molecular beacon SARS-CoV 3'-NCR MB-1 (Table 10) for the amplification and real-time detection of SARS-CoV RNA extracted from a dilution series of cultured SARS-CoV. (Figure 42) SARS-CoV 3'-NCR P1.5/P2.5; (Figure 43) SARS-CoV 3'-NCR P1.5/P2.6; (Figure 44) SARS-CoV 3'-NCR P1.5/P2.7; (Figure 45) SARS-CoV 3'-NCR P1.6/P2.5; (Figure 46) SARS-CoV 3'-NCR P1.6/P2.6; (Figure 47) SARS-CoV 3'-NCR P1.6/P2.7.

Figures 48-51 show the analysis of SARS-CoV genomic RNA, strain Frankfurt, with different primer/beacon mixtures. RNA was extracted from cultured SARS-CoV and amplified with 5 different SARS-CoV primer/beacon mixtures (Table 11). (Figure 48) SARS-CoV REP-1; (Figure 49) SARS-CoV REP-2; (Figure 50) SARS-CoV N-1; (Figure 51) SARS-CoV 3'-NCR.

Figures 52-55 show an analysis of SARS-CoV genomic RNA (Rotterdam preparation) with different primer/beacon mixtures. RNA was extracted from cultured SARS-CoV and amplified with 5 different SARS-CoV primer/beacon mixtures (Table 11). (Figure 52) SARS-CoV REP-1; (Figure 53) SARS-CoV REP-2; (Figure 54) SARS-CoV N-1; (Figure 55) SARS-CoV 3'-NCR.

Figures 56-60 show an analysis of SARS-CoV genomic RNA (Rotterdam preparation) with different primer/beacon mixtures. RNA was extracted from cultured SARS-CoV and amplified with 5 different SARS-CoV primer/beacon mixtures (Table 11). (Figure 56) SARS-CoV REP-1; (Figure 57) SARS-CoV REP-2; (Figure 58) SARS-CoV N-1; (Figure 59) SARS-CoV N-2; (Figure 60) SARS-CoV 3'-NCR.

EXAMPLES

5 First set of examples related to RNA issued from the viral replicase gene:

Example 1: Materials and Methods:

1. SARS CoV *in vitro* RNA:

10 RNA encompassing 190 nucleotides of the SARS CoV replicase gene was synthesized *in vitro* by transcription of a cloned fragment of the viral gene, essentially as described in Drosten et al. (10). The concentration of the *in vitro* generated RNA was determined by OD (260 nm) measurement and appropriate
15 serial dilutions in water were stored at -70°C until further use.

2. Oligonucleotides used for amplification and detection:

20 Primers and a molecular beacon detection probes (Tyagi, S. and Kramer, F.R. (16)) were derived from the sequence of the open reading frame (ORF) 1b of the SARS CoV replicase gene and ordered as HPLC-purified oligonucleotides (Eurogentec, Belgium). Sequence numbers and genome locations of the oligonucleotides used in the amplification and of the molecular
25 beacon probe used for specific detection in real-time, are shown in Table 1.

Table 1: Primers and molecular beacon probes for the amplification and detection in real-time of a region located in the replicase gene of SARS Coronavirus.

5

Primer/probe name	Sequence number	Primer/probe	Location
SARS-CoV REP P1.1	SEQ ID 10	Downstream primer 1.1	18319-18344
SARS-CoV REP P1.2	SEQ ID 11	Downstream primer 1.2	18283-18305
SARS-CoV REP P1.3	SEQ ID 9	Downstream primer 1.3	18389-18416
SARS-CoV REP P2.1	SEQ ID 6	Upstream primer 2.1	18153-18176
SARS-CoV REP P2.2	SEQ ID 7	Upstream primer 2.2	18201-18220
SARS-CoV REP P2.3	SEQ ID 8	Upstream primer 2.3	18221-18241
SARS-CoV REP MB-1	Label + SEQ ID 13 + quencher	Probe MB-1	18246-18271
SARS-CoV REP MB-2	Label + SEQ ID 45 + quencher	Probe MB-2	18216-18235

The two molecular beacons (SARS CoV MB-1 and MB-2) were designed for the detection of the amplicons that can be generated with the SARS-CoV REP primers outlined in Table 1. Correct folding of the molecular beacons was checked using mfold (17) web server (<http://www.bioinfo.rpi.edu/applications/mfold>). Predicted secondary structures for SARS-CoV REP MB-1 and SARS-CoV REP MB-2 are shown in Figure 6. Under NASBA reaction conditions, i.e.

41°C; 100 mM Na⁺; 12 mM Mg²⁺, both molecular beacons revealed the desired hairpin structure as the most favourable conformation. For SARS-CoV REP MB-1, additional hybrid formation was predicted within the loop portion, resulting in two other possible conformations.

Molecular beacon syntheses were analysed for their purity by capillary electrophoresis (CE). Molecular beacons have a purity varying between 80,3% for SARS-CoV REP MB-2 and 88,7% for SARS-CoV REP MB-1.

In figure 6, the arm sequences at the 5'-end and at the 3'-end are hybridized and here below depicted in small characters. Thus each probe is constituted as a molecular beacon, preferably consisting of:

SEQ ID 13: 5'-[6-FAM]-ccatgggCTGTCATGCAACTAGAGATGCTGTcccatgg-[DabSyl]-3', or

SEQ ID 45: 5'-[6-FAM]-cgcgatGTTCGTGCGTGGATTGGCTTatcgcg-[DabCyl]-3'.

Fluorophore 6-FAM is covalently linked to the 5'-end of the molecular beacon; the quenching moiety DabSyl or DabCyl is covalently linked to the 3'-end of the molecular beacon. Coordinates for the location of the hybridising segments of the primers and the molecular beacon probe are derived from the complete genome sequence of SARS coronavirus TOR2 (EMBL Accession number AY274119).

3. NASBA amplification with real-time detection:

RNA amplifications were performed using the NASBA amplification technology. To set up a NASBA amplification reaction, NucliSens Basic Kit Amplification Reagents (bioMérieux bv, The Netherlands) were used. A premix was generated by reconstituting a Reagent Sphere in a mixture of 80 µl Reagent Sphere Diluent, 18 µl KCl stock solution and 12 µl NASBA Water. Subsequently, 10 µl of a mix containing two primers for amplification and a molecular beacon probe for

real-time detection, were added, resulting in 2 x reaction buffer. Ten μ l of this reaction buffer was added to 5 μ l nucleic acid solution and incubated during 5 minutes at 65°C.

5 Then the reaction tubes were incubated at 41°C during 5 minutes. Meanwhile, an Enzyme Sphere was reconstituted in 55 μ l Enzyme Diluent and 5 μ l of the resulting enzyme mix was added into each reaction tube. After addition of the enzyme mix, tubes were mixed by gentle tapping, centrifuged briefly to
10 bring the contents to the bottom of the tubes and finally incubated at 41°C during 90 minutes in a NucliSens EasyQ Analyzer. Fluorescence in the individual reaction tubes was monitored over time and measured at regular intervals of 45 seconds.

15

Example 2: Selection of primer pair for the amplification of SARS CoV RNA:

To select a well performing primer pair for the
20 amplification of SARS CoV RNA using NASBA, three upstream primers, designated P2.1, P2.2 and P2.3, and three downstream primers , designated P1.1, P1.2 and P1.3, were derived from the sequence of the open reading frame (ORF) 1b of the SARS CoV replicase gene. Consequently, nine different primer pairs could
25 be formed, each consisting of a combination of one of the upstream primers with one of the downstream primers. With each of these primer pairs a standard dilution series of in vitro generated SARS CoV RNA was evaluated.

Primer pairs that can be formed with the SARS-CoV REP
30 primers (Table 1) are shown in Table 2 together with the size and genome location of the resulting amplicons. Results are shown in Figures 1-4 and Figures 7-11.

Table 2: SARS-CoV REP primer combinations:

Primer Combination	Amplicon Size	Genome Location
SARS-CoV REP P1.1/P2.1	192 nt	18,153- 18,344
SARS-CoV REP P1.1/P2.2	144 nt	18,201- 18,344
SARS-CoV REP P1.1/P2.3	124 nt	18,221- 18,344
SARS-CoV REP P1.2/P2.1	153 nt	18,153- 18,305
SARS-CoV REP P1.2/P2.2	105 nt	18,201- 18,305
SARS-CoV REP P1.2/P2.3	85 nt	18,221- 18,305
SARS-CoV REP P1.3/P2.1	264 nt	18,153- 18,416
SARS-CoV REP P1.3/P2.2	216 nt	18,201- 18,416
SARS-CoV REP P1.3/P2.3	196 nt	18,221- 18,416

5

Figures 1-4 (for the first four pairs P1.1/P2.1, P1.1/P2.2, P1.2/P2.1 and P1.2/P2.2) show that all primer pairs revealed a positive result for the different amounts of in vitro RNA that were tested. However, analysis of the moment at which the different reactions start to reveal fluorescence signals that are well above the background signal showed that primer combination P2.2/P1.1 has strikingly lower Time-To-Positivity (TTP) values as the other primer combinations.

10

Therefore, oligonucleotide primers P1.1 and P2.2 were selected as the most ideal primer pair for the amplification of SARS CoV RNA.

5 In a next step, all nine SARS-CoV REP primer pairs (Table 2) were analysed. Since P1.3 was chosen outside the fragment covered by the *in vitro* RNA, these primer combinations were analysed on RNA extracted from a cultured SARS-CoV virus stock (provided by Robert Koch Institute, Berlin, Germany). A nucleic acid extract was prepared from 5 µl of this virus stock containing about 5000 genome equivalents (geq). From this nucleic acid extract a ten-fold dilution series in water was prepared containing nucleic acid levels as if extracted from 5000 geq (undiluted extract) down to 0.01 geq. Each of the nine SARS-CoV REP primer combinations (see Table 2) was tested with this dilution series in real-time NASBA reactions using molecular beacon SARS-CoV REP MB-1 for detection. In addition to Figures 1-4, results are shown in Figure 7-11 for the other primer pairs (respectively for the following pairs P1.1/P2.3, P1.2/P2.3, P1.3/P2.1, P1.3/P2.2 and P1.3/P2.3).

20 The four primer combinations previously tested on *in vitro* RNA, revealed a similar result with viral RNA extracted from the cultured SARS-CoV virus stock (Figures 1-4). Regarding sensitivity of the primer combinations, some minor differences were observed, but whether these are significant, cannot be concluded on the basis of this limited evaluation. However, again primer pair P1.1/P2.2 showed faster kinetics and shorter time-to-positivity values as the other three primer combinations.

30 For some of the new primer combinations containing either P1.3 or P2.3, sensitivity was comparable to the sensitivity of primer combination SARS-CoV REP P1.1/P2.2. In addition, primer combination SARS-CoV REP P1.1/P2.3 (Figure 7) showed kinetics and time-to-positivity values similar to those of primer combination P1.1/P2.2. Strikingly, primer combination SARS-CoV

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REP P1.2/P1.3 (Figure 8) revealed a much lower sensitivity as any of the other primer pairs. Probably, this is due to the small size of only 85 nts for the amplicons generated by this primer pair (see Table 2). For NASBA amplification, an amplicon size below 100 nts is considered sub optimal. With 264 nts, primer pair SARS-CoV REP P1.3/P2.1 revealed the longest amplicons. This might explain the significantly lower kinetics, especially for the lower input levels, observed for this primer combination (Figure 9).

From the nine possible primer combinations, more or less arbitrarily, primer combination SARS-CoV REP P1.1/P2.2 was selected for further evaluation and in combination with molecular beacon SARS-CoV REP MB-1 will be referred to as primer/beacon mix SARS-CoV REP-1.

Another primer combination of interest in this region of the SARS-CoV genome, primer pair SARS-CoV REP P1.3/P2.3 in combination with molecular beacon SARS-CoV REP MB-1 was selected as SARS-CoV REP-2 for further studies.

Example 3: Analytical sensitivity of the selected primer pair:

To determine the analytical sensitivity of the selected primer pair (P1.1/P2.2) a dilution series of SARS CoV in vitro RNA was tested with the selected primer pair. The RNA dilution series was made in NASBA water from a 1×10^5 copies/ μ l RNA stock solution. Results are summarized in Figure 5 and show a very good analytical sensitivity with positive results down to 2.5 copies of in vitro RNA in de amplification, while the negative control remained negative. In present evaluation, sensitivity of the selected primer pair (P1.3/P2.3) appeared somewhat less (one ten-fold dilution) as for the selected primer pair (P1.1/P2.2), but this is not considered to be a significant difference.

Example 4: SARS CoV REP-1 "ECL" version:

Based on the primers SARS-CoV P1.1.P2.2 primers and
5 molecular beacon MB-1, primers and a capture probe were
designed that could be used to perform real-time NASBA
amplification and subsequent end-point detection by
ElectroChemiluminescence hereafter called ECL (18). Therefore,
the Basic Kit ECL-tail (Deiman, B. et al. (19)) was added to
10 the 5'-end of SARS-CoV REP P2.2 and the hybridizing fragment of
molecular beacon SARS-CoV REP MB-1 was synthesized as a
biotinylated oligonucleotide resulting in SARS-CoV REP 2.2
"ECL" and SARS-CoV REP Cap-1 (Capture probe), respectively.
Sequences, polarity, and genome locations of SARS-CoV REP-1
15 "ECL" are outlined in Table 3. For the P1 primer (SARS-CoV REP
P1.1) the T7 polymerase promoter sequence is depicted in small
characters. For the P2 primer (SARS-CoV REP P2.2 "ECL") the ECL
tail sequence added at the 5'-end of the primer is depicted in
small characters. The SARS-CoV Capture probe carries a biotin
20 moiety at its 5'-end. Coordinates for the location of the
hybridizing segments of the primers and the capture probe are
derived from the genome sequence of SARS-CoV strain TOR2
(EMBL/GenBank Accession Number AY274119).

Table 3: Primers and biotinylated capture probe for the amplification and ECL-based end-point detection of a region located in the replicase gene of SARS-CoV.

Description	Sequence (SEQ ID number)	Length	Genome location	Purity (CE)
SARS-CoV REP P.1.1	5'- aattctaatacgactcactatagggAACATAACCAG TCGGTACAGCTACTA-3' (SEQ ID 4)	51 nt	18,319- 18,344	74,1%
SARS-CoV REP P2.2 ECL	5'-gatgcaaggtcgcatatgagGAAGCTATTCGTCA CGTTCG-3' (SEQ ID 7)	40 nt	18,201- 18,220	88,6%
SARS-CoV REP Cap-1	5'-(Biotin)-GCTGTCATGCAACTAGAGATG CTGT-3' (SEQ ID 46)	21 nt	18,246- 18,271	ND

Primer/beacon mix "ECL" (containing primers SARS-CoV REP P1.1 and SARS-CoV REP P2.2 "ECL" in combination with SARS-CoV REP MB-1) was tested in real-time NASBA reactions using a dilution series of *in vitro* RNA (18) as input material in the reactions. For comparison, the same dilution series was amplified with the standard SARS-CoV REP-1 primer/beacon mix. Subsequently, ECL detection was performed on the SARS-CoV REP P1.1 and SARS-CoV REP P2.2 in combination with SARS-CoV REP MB-1 reactions, allowing direct comparison of ECL end-point detection versus real-time molecular beacon detection on the same amplification reactions. Results are summarized in Table 4, reflecting fluorescence signals for SARS-CoV REP-1 and both fluorescence signals and ECL counts for SARS-CoV REP-1 "ECL". The typical "all-or-nothing" results as obtained for SARS-CoV REP-1 were also obtained for the ECL-version of this primer/beacon mix and sensitivity of both primer/beacon mixtures is identical. Comparison of real-time versus ECL results for SARS-CoV REP-1 "ECL" revealed a similar result: ECL counts were either very low or "overrange" ($> 10^7$ counts) and

dilutions found positive with real-time detection were positive with ECL detection as well.

- 5 Table 4: Comparison of SARS-CoV REP-1 primer/beacon mixtures and real-time versus ECL detection.

SARS CoV REP-1	
RNA input (cps per reaction)	Fluorescence signal
10	9,86
5	9,45
1	9,36
0,5	9,53
0,1	9,02
0,05	8,98
0,01	1,19
No Template	1,19

SARS CoV REP-1 "ECL"		
RNA input (cps per reaction)	Fluoresce nce signal	ECL signal (counts)
10	8,61	10.000.00 1
5	8,87	10.000.00 1
1	8,97	10.000.00 1
0,5	9,18	10.000.00 1
0,1	8,67	10.000.00 1
0,05	8,69	10.000.00 1
0,01	1,15	171
No Template	1,17	172

5 *In vitro* RNA was amplified with primer/beacon mixtures SARS-CoV REP-1 and SARS-CoV REP-1 "ECL". For subsequent ECL detection, SARS-CoV REP Cap-1 was used in combination with the generic ECL probe of the NucliSens Basic Kit.

10 Second set of examples related to RNA issued from the viral gene encoding the nucleocapsid protein:

Example 5: SARS-CoV Nucleocapsid primers design:

15 The SARS-CoV Nucleocapsid (N) protein is encoded by the open reading frame spanning nucleotides 28,120-29,388 on the viral genome. Primers were designed for two regions in the nucleocapsid gene, the first region encompassing nucleotides

28,251-28,485 (region 1) and the second region spanning nucleotides 28,851-29,101 (region 2). For each region, initially two P1 primers (SARS-CoV N P1.1 and P1.2 for region 1; SARS-CoV N P1.3 and P1.4 for region 2) and two P2 primers (SARS-CoV N P2.1 and P2.2 for region 1; SARS-CoV N P2.3 and P2.4 for region 2) were designed. In each of the regions an additional P2 primer was chosen that hybridized closer to the sequence targeted by the molecular beacon for that region (SARS-CoV N P2.5 for region 1; SARS-CoV N P2.6 for region 2). Consequently, smaller amplicons could be generated which might have a positive impact on the sensitivity of SARS-CoV N subgenomic RNA detection. Sequences, polarity, and genome locations of all SARS-CoV N primers are shown in Table 5.

Table 5: Primers for the amplification in real-time of two regions located in the Nucleocapsid gene of SARS-CoV.

Region 1

Description	Sequence (SEQ ID number)	Length	Genome Location	Purity (CE)
SARS-CoV N P1.1	5'- aattctaatacgcactcactatagggAGAAGTACCA TCT GGGGCTGA-3' (T7 + SEQ ID 15)	46 nt	28,433- 28,453	85,5%
SARS-CoV N P1.2	5'- aattctaatacgcactcactatagggAAGTGAAGCT TCT GGGCCAGTTCCTA-3' (T7 + SEQ ID 16)	51 nt	28,460- 28,485	85,1%
SARS-CoV N P2.1	5'-AGGTTTACCCAATAATACTGCGT-3' (SEQ ID 18)	23 nt	28,251- 28,273	90,2%
SARS-CoV N P2.2	5'-AGATTCCCTCGAGGCCAGGGCGT-3' (SEQ ID 19)	23 nt	28,315- 28,337	91,5%
SARS-CoV N P2.5	5'-ATAGTGGTCCAGATGACCAAAT-3' (SEQ ID 20)	22 nt	28,352- 28,373	90,0%

Region 2

<i>Description</i>	<i>Sequence (SEQ ID number)</i>	<i>Length</i>	<i>Genome Location</i>	<i>Purity (CE)</i>
SARS-CoV N P1.3	5'- aattctaatacgactcactatagggAAGAATGCAGAG GCA CTTGGAGCA-3' (SEQ ID 41)	49 nt	29,043- 29,066	82,1%
SARS-CoV N P1.4	5'- aattctaatacgactcactatagggAAGGTGTGACTT CCATGC CAA-3' (SEQ ID 42)	46 nt	29,081- 29,101	81,8%
SARS-CoV N P2.3	5'-CCAAACTGTCTACTAAGAAATCTGCT-3' (SEQ ID 27)	25 nt	28,851- 28,875	87,5%
SARS-CoV N P2.4	5'-CTCAAGCATTTGGGAGACGTGGT-3' (SEQ ID 28)	23 nt	28,934- 28,956	82,8%
SARS-CoV N P2.6	5'-CAGAACAAACCCAAGGAAATT-3' (SEQ ID 29)	21 nt	28,958- 28,978	89,8%

For the P1 primers (SARS-CoV N P1.1, P1.2, P1.3 and P1.4) the T7 Polymerase promoter sequence is depicted in small characters. Coordinates for the location of the primers are derived from the complete genome sequence of SARS-CoV isolate TOR2 (GenBank Accession number AY274119). With two P1 primers and three P2 primers, six different SARS-CoV N primer combinations can be composed for each of the two regions (Table 6).

Table 6: SARS-CoV N primers combinations.

<i>Primer Combination for region 1</i>	Amplicon Size	Genome Location
SARS-CoV N P1.1/P2.1	203 nt	28,251-28,453
SARS-CoV N P1.1/P2.2	139 nt	28,315-28,453
SARS-CoV N P1.1/P2.5	102 nt	28,352-28,453
SARS-CoV N P1.2/P2.1	235 nt	28,251-28,485
SARS-CoV N P1.2/P2.2	171 nt	28,315-28,485
SARS-CoV N P1.2/P2.5	134 nt	28,352-28,485

<i>Primer Combination for region 2</i>	Amplicon Size	Genome Location
SARS-CoV N P1.3/P2.3	216 nt	28,851-29,066
SARS-CoV N P1.3/P2.4	133 nt	28,934-29,066
SARS-CoV N P1.3/P2.6	109 nt	28,958-29,066
SARS-CoV N P1.4/P2.3	251 nt	28,851-29,101
SARS-CoV N P1.4/P2.4	168 nt	28,934-29,101
SARS-CoV N P1.4/P2.6	144 nt	28,958-29,101

Example 6: SARS-CoV Nucleocapsid molecular beacons design:

For the detection of the amplicons that can be generated with the different SARS-CoV N primers outlined in Table 5, a molecular beacon was designed for each of the two selected target regions in the N gene. The molecular beacons were designated SARS-CoV N MB-1 for the upstream (region 1) region and SARS-CoV N MB-2 for the downstream region (region 2). Correct folding of the molecular beacons was checked using mfold (17). Predicted secondary structures for SARS-CoV N MB-1 and SARS-CoV N MB-2 are shown in Figure 12. The mfold web server (<http://www.bioinfo.rpi.edu/applications/mfold/>) was used to predict the most preferred secondary structures for molecular beacons SARS-CoV N MB-1 and SARS-CoV N MB-2. Under NASBA reaction conditions [41°C; 100 mM Na⁺; 12 mM Mg²⁺], both molecular beacons revealed the desired hairpin structure. Molecular beacon syntheses were analysed for their purity by capillary electrophoresis (CE). Molecular beacon features are summarized in Table 7.

Table 7: Molecular beacon probes for the detection in real-time of two regions located in the Nucleocapsid gene of SARS-CoV.

Description	Sequence (SEQ ID number)	Length	Genome Location	Purity (CE)
SARS-CoV N MB-1	5'-(6-FAM)- ccatgggCTACTACCGAAGAGCTACCCG ACGAcccatgg-(DabSyl)-3' (label + SEQ ID22 + quencher)	39 nt	28,377- 28,401	88,2%
SARS-CoV N MB-2	5'-(6-FAM)- ccatggACCAAGACCTAATCAGACAacatgg- (DabSyl)-3' (label + SEQ ID 30 + quencher)	32 nt	28,985- 29,004	75,5%

For the molecular beacons (SARS-CoV N MB-1 and MB-2) the arm sequences at the 5'-end and at the 3'-end are depicted in small characters. Fluorophore 6-FAM is covalently linked to the 5'-end of the molecular beacons; the quenching moiety DabSyl is covalently linked to the 3'-end of the molecular beacons. Coordinates for the location of the molecular beacon probes are derived from the complete genome sequence of SARS-CoV isolate TOR2 (GenBank Accession number AY274119).

Example 7: Selection of primer pair/beacon combination for the amplification and detection of SARS CoV N RNA:

For each of the regions chosen for amplification in the SARS-CoV Nucleocapsid gene sequence, six different primer combinations can be composed with the primers shown in Table 5. In Table 6, all combinations are summarized, together with the size of the corresponding RNA amplicons.

Initially, four SARS-CoV N primer pairs for region 1 that could be formed with P1.1, P1.2, P2.1, and P2.2 (Table 6) were tested in real-time NASBA reactions using molecular beacon SARS-CoV N MB-1 labelled with FAM (Table 7) for detection. Input RNA for these reactions was prepared from a cultured SARS-CoV virus stock (provided by Robert Koch Institute, Berlin, Germany) by extracting nucleic acid from a ten-fold dilution series containing 10,000 down to 0.1 geq of the virus. Results are shown in Figures 13-16. Combined with either of the P2 primers, SARS-CoV N P1.1 revealed a better performance as P1.2 (compare Figures 13 and 14 with Figures 15 and 16), although not in terms of sensitivity but with respect to kinetics and time-to-positivity values for the different dilutions.

In a follow-up experiment, the different primer combinations were compared again. Similar nucleic acid extracts were used as input material although the isolates were not

prepared freshly as in the previous experiment (Figures 13-16), but had been stored at -70°C for a while. Results are summarized in Figures 17-20. For reasons not understood, poor results were obtained for the nucleic acid extract originating from 100 geq. Nevertheless, relative comparison of the different primer combinations again identified P1.1 as the best performing P1 primer. Combination of this P1 primer with P2.2 appeared to be the most favourable primer pair because SARS-CoV N P1.1/P2.2 was the combination capable of most efficiently detecting the nucleic acid extract resulting from 1 geq and to reveal a positive albeit low signal for the "100 geq" nucleic acid extract (Figure 18) that unexpectedly tested negative for the other primer combinations.

In a comparable way as for region 1, four primer combinations for region 2 that can be compiled from primers SARS-CoV N P1.3, P1.4, P2.3, and P2.4 (Table 6) were analysed, now using FAM-labelled molecular beacon SARS-CoV N MB-2 (Table 7) for real-time detection. Results for freshly prepared nucleic acid extracts are shown in Figures 21-24. In contrast to the primer pairs for region 1 in which a P1 primer was the dominating factor, for region 2 a P2 primer, viz. SARS-CoV N P2.4, appeared to be the crucial primer. It revealed a much better performance as the other P2 primer (compare Figures 21 and 23, 22 and 24), while combination with either SARS-CoV N P1.3 or P1.4 did not make too much of a difference (see Figures 22 and 24). Although kinetics for the "10 geq" nucleic acid extract for SARS-CoV N P1.3/P2.4 were slower as for the SARS-CoV N P1.4/P2.4 primer combination, the low but evidently positive signal for the next dilution, i.e. the "1 geq" nucleic acid extract, (Figure 22) pleads in favour of the former primer combination.

Analysis of stored nucleic acid extracts containing genomic viral RNA with the region 2 primer combinations confirmed the results as obtained for the fresh specimens. As

can be seen from Figures 25-28, again P2.4 appeared to be the key primer. Combination with either P1.3 or P1.4 revealed similar results although the nucleic acid extract resulting from 1 geq was missed by the SARS-CoV N P1.3/P2.4 primer pair whereas the next dilution in line was well detected. With all four primer combinations, poor results were obtained for the "100 geq" nucleic acid extract, confirming the poor results for this isolate with the region 1 primer combinations (Figures 17-20).

In conclusion, this first endeavour to come and get to a well performing primer pair for the detection of SARS-CoV subgenomic mRNA encoding the nucleocapsid protein revealed SARS-CoV N P1.1/P2.2 in region 1 and either SARS-CoV N P1.3/P2.4 or SARS-CoV N P1.4/P2.4 in region 2 as the most likely candidates. Overall comparison of the three primer combinations identified SARS-CoV N P1.1/P2.2 (region 1) in combination with SARS-CoV N MB-1 as the most favourable primer pair, mainly based on the shape of the fluorescence curves and the absolute fluorescence signals that could be obtained with this primer/beacon mixture. Therefore, this primer/beacon mixture was selected as SARS-CoV N-1 for further evaluation.

In an attempt to further improve the sensitivity of SARS-CoV N mRNA detection, an additional P2 primer was designed for both regions, viz. SARS-CoV N P2.5 for region 1 and SARS-CoV N P2.6 for region 2 (Table 5). In either case, this new P2 primer was located closer to the P1 primer resulting in a smaller amplicon size as obtained for the initial primer combinations (Table 6).

The six SARS-CoV N primer pairs that could now be formed for each of the two regions (Table 6) were tested in real-time NASBA reactions using the FAM-labelled SARS-CoV N molecular beacon designed for each of the regions (Table 7) for detection. Input RNA for these reactions was prepared from a cultured SARS-CoV virus stock (provided by Erasmus Medical

Center, Rotterdam, The Netherlands). Nucleic acid was extracted from a 100-fold dilution of the original virus stock. From the resulting nucleic acid extract, a ten-fold dilution series was prepared containing nucleic acid as if extracted from 10^3 -fold down to 10^9 -fold dilutions of the original virus stock.

Results for the SARS-CoV N primer pairs in region 1 are shown in Figures 29-34. Remarkably, primer combinations containing the new SARS-CoV N P2.5 primer (Figures 31 and 34) demonstrated a significantly lower sensitivity as the other primer pairs instead of an anticipated better performance. This was somewhat unexpected since this P2 primer revealed shorter amplicons (102 nts and 134 nts in combination with P1.1 or P1.2, respectively; see also Table 6) and, therefore, was expected to result in more efficient amplification. Comparison of the remaining primer combinations confirmed the already observed superiority of SARS-CoV N P1.1 over SARS-CoV N P1.2. However, in contrast to earlier observations (Figures 13-20), now SARS-CoV N P2.1 appeared to be superior over SARS-CoV N P2.2, which again would not be logical in terms of amplicon length (203 nts versus 139 nts for P2.1 and P2.2, respectively; see also Table 6). The overall conclusion of this comparison is twofold:

for each of the two P1 primers: the larger the amplicon size, the better the performance of the underlying primer combination; and

P1.1 revealed a better performance as P1.2.

Results for region 2 including the additional P2.6 primer are shown in Figures 35-40. When combined with SARS-CoV N P1.3, the new P2 primer did not reveal the anticipated higher sensitivity (Figure 37), but instead showed a somewhat poorer performance as the SARS-CoV N P1.3/P2.4 primer pair both in terms of sensitivity and in time-to-positivity values for the individual dilutions. However, in combination with the other P1 primer chosen in this region and resulting in primer pair SARS-

CoV N P1.4/P2.6, sensitivity indeed increased, reflected by the result obtained for the nucleic acid extract obtained for the 10^9 dilution of the virus stock that only tested positive with this region 2 primer pair (Figure 40).

Next to SARS-CoV N P1.4/P2.6 and in line with previous observations (see Figures 21-28), also SARS-CoV N P1.3/P2.4 (Figure 36) and SARS-CoV N P1.4/P2.4 (Figure 39) again were identified as better performing primer combinations in this region and again these primer combinations mutually were nearly indistinguishable.

Third set of examples related to RNA encompassing the 3'-non coding region (3'-NCR):

Example 8: SARS-CoV 3'-NCR primers design:

The subgenomic mRNA transcripts of SARS-CoV form a 3'-co-terminal nested set with the viral genome and, consequently, have a common 3'-end sequence. Excluding the poly(A)-tail, this 3'-untranslated or non-coding region (3'-NCR) is 339 nucleotides in length and encompasses genomic positions 29,389-29,727.

Primers were designed for the amplification of fragments of the 3'-NCR between genomic nucleotides 29,447-29,713. Sequences, polarity, and genomic locations of these SARS-CoV 3'-NCR primers are shown in Table 8.

Table 8: Primers for the amplification in real-time of a region located in the 3'-untranslated sequence of SARS-CoV.

<i>Description</i>	<i>Sequence (SEQ ID number)</i>	<i>Length</i>	<i>Genome Location</i>	<i>Purity (CE)</i>
SARS-CoV 3'-NCR P1.5	5'- aattctaatacgactcactatagggGGGCTCTT CCATATA GGCA-3' (T7 + SEQ ID 32)	44 nt	29,642- 29,660	83,3%
SARS-CoV 3'-NCR P1.6	5'- aattctaatacgactcactatagggAAGCTATT AAAATC ACATGGGGA-3' (T7 + SEQ ID 33)	48 nt	29,691- 29,713	85,6%
SARS-CoV 3'-NCR P2.5	5'-TACGATACATAGTCTACTCTTGT-3' (SEQ ID 35)	23 nt	29,447- 29,469	91,7%
SARS-CoV 3'-NCR P2.6	5'-TAACTAAACAGCACAAAGTAGGT-3' (SEQ ID 36)	22 nt	29,486- 29,507	86,5%
SARS-CoV 3'-NCR P2.7	5'-TAGCAATCTTTAATCAATGT-3' (SEQ ID 37)	20 nt	29,529- 29,548	91,3%

5

For the P1 primers (SARS-CoV 3'-NCR P1.5 and P1.6) the T7 Polymerase promoter sequence is depicted in small characters. Coordinates for the location of the primers are derived from the complete genome sequence of SARS-CoV isolate TOR2 (GenBank Accession number AY274119).

10

Two P1 primers (SARS-CoV 3'-NCR P1.5 and P1.6) and three P2 primers (SARS-CoV 3'-NCR P2.5, P2.6, and P2.7) were derived from the viral genome sequence and enabled the composition of six different primer pairs (Table 9).

15

Table 9: SARS-CoV 3'-NCR primer combinations.

Primer Combination	Amplicon Size	Genome Location
SARS-CoV 3'-NCR P1.5/P2.5	214 nt	29,447- 29,660
SARS-CoV 3'-NCR P1.5/P2.6	175 nt	29,486- 29,660
SARS-CoV 3'-NCR P1.5/P2.7	132 nt	29,529- 29,660
SARS-CoV 3'-NCR P1.6/P2.5	267 nt	29,447- 29,713
SARS-CoV 3'-NCR P1.6/P2.6	228 nt	29,486- 29,713
SARS-CoV 3'-NCR P1.6/P2.7	185 nt	29,529- 29,713

5

Primer pairs that can be formed with the SARS-CoV 3'-NCR primers (Table 8) are given together with the size and genome location of the resulting amplicons.

10 **Example 9: SARS-CoV 3'-NCR molecular beacon design:**

Molecular beacon SARS-CoV 3'-NCR MB-1 was designed as shown in Table 10 for the detection of amplicons that can be generated with the SARS-CoV 3'-NCR primers outlined in Table 8.

Table 10: Molecular beacon for the detection in real-time of amplicons generated from a region located in the 3'-untranslated sequence of SARS-CoV.

<i>Description</i>	<i>Sequence</i>	<i>Length</i>	<i>Genome Location</i>	<i>Purity (CE)</i>
SARS-CoV 3'-NCR MB-1	5'-(6-FAM)- ccatgcGCCACCACATTTTCATCGAG gcatgg-(DabSyl)-3' (label + SEQ ID 47 + quencher)	32 nt	29,574- 29,595	84,6%

For the molecular beacon (SARS-CoV 3'-NCR MB-1) the arm sequences at the 5'-end and at the 3'-end are depicted in small characters. Fluorophore 6-FAM is covalently linked to the 5'-end of the molecular beacon; the quenching moiety DabSyl is covalently linked to the 3'-end of the molecular beacon. Coordinates for the location of the molecular beacon probe are derived from the complete genome sequence of SARS-CoV isolate TOR2 (GenBank Accession number AY274119).

Correct folding of the molecular beacon was checked using mfold (17). The secondary structure as predicted for SARS-CoV 3'-NCR MB-1 is shown in Figure 41. Under NASBA reaction conditions [41°C; 100 mM Na⁺; 12 mM Mg²⁺], the molecular beacon revealed the desired hairpin structure.

Synthesis of the molecular beacon was analysed by capillary electrophoresis (CE) and revealed a purity of about 84.6% (Table 10).

Example 10: Selection of primer pair/beacon combination for the amplification and detection of SARS-CoV 3'-NCR:

Six different primer combinations could be formed with the SARS-CoV 3'-NCR primers shown in Table 8. Primer combinations are depicted in Table 9 together with the size of the resulting RNA amplicons.

The different SARS-CoV 3'-NCR primer pairs were combined with the FAM-labelled SARS-CoV 3'-NCR molecular beacon (Table 10) and tested in real-time NASBA reactions. Input RNA for these reactions was prepared from a cultured SARS-CoV virus stock (provided by Robert Koch Institute, Berlin, Germany). From a nucleic acid extract prepared from 1000 geq of the virus, a ten-fold dilution series in water was prepared. This dilution series contained nucleic acid levels as if extracted from 100 geq down to 0.01 geq.

Results for the six primer combinations are shown in Figures 42-47 and do not identify a preferred P1 or P2 primer but rather two unrelated primer combinations being SARS-CoV 3'-NCR P1.5/P2.5 and P1.6/P2.6 (Figures 42 and 46 respectively). For the other primer combinations, a lower sensitivity (SARS-CoV 3'-NCR P1.5/P2.6; Figure 43) or poorer kinetics, especially for the lower input levels (Figures 44, 45 and 47), were observed.

Showing "steeper" and faster (i.e. shorter TTP values) kinetics, primer pair SARS-CoV 3'-NCR P1.6/P2.6 was chosen for further evaluation and in combination with molecular beacon SARS-CoV 3'-NCR MB-1 will be referred to as primer/beacon mix SARS-CoV 3'-NCR.

Comparison of SARS-CoV primer/molecular beacon mixtures for different regions of the genome:

Upon analysis of several different primer combinations in three regions of the viral genome, viz. the replicase gene, the nucleocapsid gene, and the untranslated 'leader' sequence at the 3'-end of the genome, the best performing primer/beacon mixtures for each of the regions were selected for mutual comparison. In Table 11 these primer/beacon mixtures and their individual components are summarized.

Table 11: SARS-CoV primer/beacon mixtures targeting different regions on the SARS-CoV viral genome and their individual constituents.

5

<i>Primer/beacon mix</i>	P1 primer	P2 primer	Molecular beacon
SARS-CoV REP-1	SARS-CoV REP P1.1	SARS-CoV REP P2.2	SARS-CoV REP MB-1
SARS-CoV REP-2	SARS-CoV REP P1.3	SARS-CoV REP P2.3	SARS-CoV REP MB-1
SARS-CoV N-1	SARS-CoV N P1.1	SARS-CoV N P2.2	SARS-CoV N MB-1
SARS-CoV N-2	SARS-CoV N P1.4	SARS-CoV N P2.6	SARS-CoV N MB-2
SARS-CoV 3'-NCR	SARS-CoV 3'-NCR P1.6	SARS-CoV 3'-NCR P2.6	SARS-CoV 3'-NCR MB-1

Example 11: Cultured SARS-CoV virus strains – first sample:

10 A first comparison of the different primer/beacon mixtures was made on viral genomic RNA extracted from a cultured SARS-CoV specimen that was kindly provided by the Robert Koch Institute in Berlin, Germany. This cultured SARS-CoV virus originated from the SARS case cluster from Frankfurt, Germany (10). Based on this virus stock with a known concentration of virus particles (in genome equivalents (geq) per ml), a ten-fold dilution series was prepared. RNA was extracted in duplicate from 2000 geq down to 0.2 geq of the virus using SAME extraction (see "Materials and Methods" section) and finally eluted in 50 µl elution buffer. Consequently, RNA resulting from 200 geq down to 0.02 geq was amplified with five different primer/beacon mixtures that were selected for further evaluation (Table 11).

Typical analyses are shown in Figures 48-51 and overall results are summarized in Table 12.

5 Table 12: Analysis of SARS-CoV genomic RNA, strain Frankfurt, with different primer/beacon mixtures (Results from two analyses were compiled).

RNA in ampl. a (geq)	SARS-CoV REP-1	SARS-CoV REP-2	SARS- CoV N-1	SARS- CoV 3' -NCR
200	2/2	2/2	2/2	2/2
20	2/2	2/2	2/2	2/2
2	2/2	2/2	2/2	1/2
0.2	2/2	2/2	2/2	2/2
0.1	1/2	0/2	2/2	0/2
0.02	0/2	0/2	0/2	0/2

a = assuming 100% recovery in nucleic acid extraction

10

SARS-CoV N-1 and SARS-CoV REP-1 and REP-2 primer/beacon mixtures revealed the highest sensitivity. SARS-CoV N-1 was the most sensitive combination although differences with the SARS-CoV REP mixtures were minimal. SARS-CoV 3'-NCR showed
15 intermediate sensitivity.

Example 12: Cultured SARS-CoV virus strains - second sample:

For a second comparison, cultured virus from a different
20 source was used. This virus stock originated from the Erasmus Medical Center in Rotterdam, The Netherlands. Concentration of virus particles in this virus preparation was unknown. Therefore, a limiting dilution series was prepared ranging from a 100-fold dilution of the original virus culture down to a
25 10⁹-fold dilution. RNA was extracted from each dilution using SAME extraction (see "Materials and Methods" section) and

amplified with each of the selected primer/beacon mixtures (Table 11). Typical analyses are shown in Figures 52-55 and overall results of three dilution series are summarized in Table 13)

Table 13: Analysis of SARS-CoV genomic RNA, strain Rotterdam, with different primer/beacon mixtures (Results from three analyses were compiled).

Virus stock dilution Factor	SARS-CoV REP-1	SARS-CoV REP-2	SARS-CoV N-1	SARS-CoV 3' -NCR
10^2	3/3	3/3	3/3	3/3
10^3	3/3	3/3	3/3	3/3
10^4	3/3	3/3	3/3	3/3
10^5	3/3	3/3	3/3	3/3
10^6	3/3	3/3	3/3	3/3
10^7	2/3	3/3	3/3	3/3
10^8	1/3	1/3	2/3	1/3
10^9	1/3	0/3	0/3	0/3
Neg	0/3	0/3	0/3	0/3

In line with the results found for the virus from Frankfurt, again SARS-CoV N-1 and SARS-CoV REP primer/beacon mixtures turned out to be the most sensitive combinations. Looking at the lower virus concentrations (10^7 - 10^9 dilutions), SARS-CoV N-1 revealed the most consistent results. However, SARS-CoV REP-1 was the only primer/beacon mixture to detect the 10^9 -fold dilution albeit in only one out of three tests.

Example 13: Cultured SARS-CoV virus strains - third sample:

For a third comparison, again cultured virus from the Erasmus Medical Center in Rotterdam, The Netherlands, was used. Concentration of virus particles in this virus preparation was unknown. Therefore, a limiting dilution series was prepared ranging from a 10^3 -fold dilution of the original virus culture down to a 10^{10} -fold dilution. RNA was extracted from each dilution using SAME extraction (see "Materials and Methods" section) and amplified with each of the selected primer/beacon mixtures (Table 11). Analyses are shown in Figures 56-60. In this comparison, SARS-CoV REP-1 (Figure 56), SARS CoV REP-2 (Figure 57) and SARS-CoV N-2 (Figure 59) appeared to be the most sensitive primer/beacon mixtures. However, the differences with SARS CoV N-1 (Figure 58) was only one 10-fold dilution. SARS CoV 3'-NCR (Figure 60) again revealed the lowest sensitivity as was also observed in the other comparisons that were made on cultured viral materials.

BIBLIOGRAPHY

- (1) Marra, M A , Jones, S J M , Astell, C R et al. The genomic sequence of the SARS-associated Coronavirus.
 5 www.sciencexpress.org / 1 May 2003 / page 1-5/10.1126/science.1085953
- (2) Rota, P A , Oberste M S, Monroe, S S et al. Characterization of a Novel Coronavirus Associated with Severe Acute Respiratory Syndrome. www.sciencexpress.org / 1 May 2003
 10 / Page 1-7/ 10.1126/science.1085952
- (3) Anonymous. Acute respiratory syndrome in China – update 3:disease outbreak reported.Geneva:World Health Organization,February 2003.
- (4) Update:outbreak of severe acute respiratory syndrome –
 15 worldwide,,2003.MMWR Morb Mortal Wkly Rep 2003;52:241-8.
- (5) Tsang KW,Ho PL,Ooi GC,et al.A cluster of cases of severe acute respiratory syndrome in Hong Kong.N Engl J Med 2003;348:1975-83.
- (6) Lee N,Hui D,Wu A,et al.A major outbreak of severe acute
 20 respiratory syndrome in Hong Kong.N Engl J Med 2003;348:1984-92.
- (7) Poutanen SM,Low DE,Henry B,et al.Identification of severe acute respiratory syndrome in Canada.N Engl J Med 2003;348:1993-2003.
- (8) Bitsch, A. et al., J Infect. Dis 167, 740-743, 1993
 25
- (9) Meyer, T. et al., Mol. Cell Probes. 8, 261-271, 1994
- (10) Drosten, C, Gunther, S, Preiser, W et al. Identification of a novel Coronavirus in patients with Severe Acute Respiratory Syndrome. N Engl J Med,2003, 348, 1967-76.
- (12) Compton J. Nucleic acid sequence based amplification –
 30 Nature 1991 Volume 350 pages 91-2.
- (13) Ksiazek,T G, Erdman,D, Goldsmith,C S et al. A Novel Coronavirus Associated with Severe Acute Respiratory Syndrome. N Engl J Med, 2003, 348, 1953-66.

- (14) Sawicki G. S. et al. Adv. Exp. Med. Biol., volume 440, from page 215, 1998.
- (15) Lai M.M.C. et al. in Fields Virology, D.M. Knipe, P.M. Howley, Eds (Lippincott Williams and Wilkins, New York, 4th edition, 2001), Ch 35.
- (16) Tyagi, S. and Kramer, K.R. (1996) Molecular beacons: probes that fluoresce upon hybridization. *Nature Biotechnology*;14:303-308.
- (17) Zuker, M. (2003) Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Research* 31, 3406-3415.
- (18) Kenten, J.H., Gudibande, S., Link, J., Willey, J.J., Curfman, B., Major, E.O., Massey, R.J. (1992) Improved Electrochemiluminescent label for DNA probe assays for HIV-1 PCR products. *Clinical Chemistry* 38, 873-879.
- (19) Deiman, B.; van Aarle, P.; and Sillekens, P. (2002) Characteristics and applications of nucleic acid sequence-based amplification (NASBA). *Molecular Biotechnology* 20, 163-179